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Engineering of sugar metabolism in *Lactococcus lactis*

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CHAPTER 1

GENERAL INTRODUCTION

Wietske A. Pool

Lactococcus lactis

Bacteria represent the oldest, most simple and most diverse life-forms on earth. They can be found everywhere on the planet and they can multiply fast even under the most harsh conditions. Up to now only a relatively low number of species has been identified (218). The species that have been identified are usually the most abundant ones, which are easy to grow in a laboratory setting.

The molecular genetic and biochemical work described in this thesis was performed on a strain of the lactic acid bacterium *Lactococcus lactis*. Lactic acid bacteria (LAB) are bacteria that can ferment a great variety of sugars to predominantly lactate in a process that is called homolactic fermentation. LAB can also be heterofermentative, producing acetate, ethanol, formate and carbon dioxide, apart from lactate. LAB are widely used in food fermentations such as in the production of cheese and yogurt, in sausage fermentation, and in the manufacture of certain wines. Well studied genera of LAB are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Streptococcus* (72). The species *L. lactis*, formerly known as lactic or group N *Streptococcus*, consists of 3 subspecies: *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *hordniae*. The first two are used in starter cultures for dairy products including all sorts of cheeses and sour products, where traits like fast acidification, production of food preservatives, production of flavour compounds, and production of exopolysaccharides for texture development are of utmost importance (35, 76). Recently, interest is also growing in some plant-associated non-dairy *L. lactis* strains, providing possible new flavour-forming traits beneficial for dairy fermentations (66).

L. lactis is also used extensively as a model organism for facultative anaerobic low-GC Gram-positive bacteria since this food-grade bacterium is easy to culture under laboratory conditions and has a relatively simple metabolism. As a representative of *L. lactis* subsp. *cremoris*, strain MG1363 is studied intensively. The model for *L. lactis* subsp. *lactis* is strain IL1403. Because of their industrial importance and their role in fundamental research, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* have been studied in great detail. The nucleotide sequence of the whole chromosome has been determined of both model strains IL1403 (15) and MG1363 (215) and also of *L. lactis* subsp. *cremoris* strain SK11 (108). This genomic information as well as the in-depth knowledge of the physiology and biochemistry of *L. lactis* has recently allowed to conduct advanced metabolic engineering strategies e.g. to obtain bacteria with nutraceutical traits. Nutraceuticals represent a wide range of foods and food components with a claimed health benefit. Examples of such beneficial strain engineering in *L. lactis* are the simultaneous overproduction of

the vitamins folate and riboflavin (183), and other industrially important features like the production of the buttery flavor diacetyl (64), or the production of the sweet amino acid L-alanine (61). The aim of the work presented in this Thesis is to gain lactococcal strains with diverse sugar uptake and metabolic capacities, focussing on the sugars glucose, galactose and lactose.

Sugar transport systems

Most bacteria have the ability to use a great variety of carbon sources as an evolutionary advantage to be able to adapt to their ever-changing habitats. The first step in bacterial carbon metabolism is the uptake of a carbohydrate, which can take place via several mechanistically different uptake routes.

First, carbohydrates can be actively imported via primary transport systems. In this type of transport the carbohydrate is imported at the expense of ATP, which is hydrolysed to ADP and inorganic phosphate (Pi). The transporters involved are called ABC-transporters, since they carry an ATP-binding cassette domain. In LAB, this type of transport system is mainly used for the internalization of amino acids and di-, tri- and oligopeptides. Although sugar transport via ABC-transporters seems not to be very common in LAB, some studies show that it is possible. In *Lactobacillus acidophilus*, the four-component ABC-transport systems of the MsmEFGK-family are involved in the uptake of the sugars raffinose and fructo-oligosaccharides (7, 8). The disaccharide maltose is transported in *L. lactis* by an ABC-transport system (90). MalEFGK₂ is a well-studied ABC-transporter involved in maltose transport in *Escherichia coli* (16, 129, 168). In archaea, most ABC-transporters characterized to date are involved in the uptake of carbohydrates, for example for the uptake of glucose (GlcSTUV), arabinose (AraSTUV), cellobiose (CbtABCDF), maltose (MalEFGK) and trehalose (TreSTUV) in *Sulfolobus solfataricus* (2, 46).

A second type of sugar transport, so-called secondary transport, is driven by the energy stored in electrochemical gradients (78). Secondary transport includes symport (a sugar is imported together with a coupling-molecule, mostly H⁺ or Na⁺), antiport (a sugar is excreted while a coupling-molecule is imported at the same time), and uniport (uptake of only a sugar molecule). Secondary sugar transport in LAB is also rarely described. The best studied example of a secondary transport protein of LAB is the lactose transporter (LacS) of *Streptococcus thermophilus* (48, 143, 145). LacS can import lactose via two mechanisms: it can internalize lactose together with H⁺, but *in vivo* the fastest reaction is the antiport of lactose and

galactose. This antiport reaction is favourable, since galactose (formed by hydrolysis of lactose in the cell) cannot be metabolized by most *S. thermophilus* strains (48, 212).

The third mechanism of carbohydrate transport in bacteria is group translocation via the phosphoenolpyruvate (PEP)-dependent : carbohydrate phosphotransferase system (PTS), which was first discovered more than forty years ago in *E. coli* (85). The PEP:PTS is a general system used by many organisms for the internalization of a great diversity of carbohydrates (42, 148, 205). The system involves uptake accompanied by phosphorylation of the transported carbohydrate. The phosphate group is donated to the carbohydrate via a phosphate transfer cascade originating from phosphoenolpyruvate (PEP) (Fig. 1).

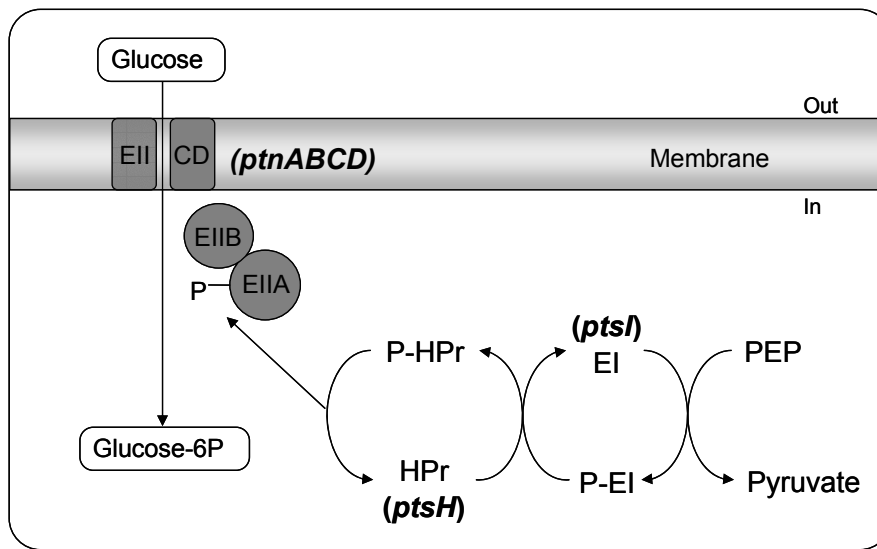


Figure 1: The phosphoenolpyruvate-dependent : carbohydrate phosphotransferase system (PEP:PTS). In this example glucose is the imported molecule, the EII-conformation of a mannose-class EII is expected (148). The phosphate cascade from phosphoenolpyruvate (PEP) via enzyme I (EI, encoded by *ptsI*), histidine protein (HPr, encoded by *ptsH*) and enzyme IIA (EIIA, encoded by part of *ptnABCD* in *L. lactis* MG1363) to the imported sugar is shown.

First, the phosphate of PEP is transferred to Enzyme I (EI), which concomitantly transfers the phosphate to the histidine residue at position 15 of the histidine protein (HPr), followed by phosphate transfer to Enzyme II (EII). EII transfers the phosphate

group to the imported carbohydrate. EI and HPr (encoded by *ptsI* and *ptsH*, respectively) are used for most of the different carbohydrates that can be imported via PEP:PTSs by a certain organism, while EII is carbohydrate-specific. The functional EII can have different domains or may consist of several separate EII proteins. EII always contains one membrane-bound protein (which may consist of different domains, e.g. IIBC), or two integral membrane-bound proteins (e.g. IIC and IID). Furthermore, one cytosolic protein (formerly called Enzyme III) consisting of one or two domains (e.g. IIA or IIAB) may be present (148).

The PEP:PTSs use one PEP molecule for the transport and concomitant phosphorylation of the imported sugar, and is energetically the most favourable sugar uptake system. The PEP molecule used in this reaction is energetically equivalent to one ATP molecule since one ATP is formed in the glycolytic reaction from PEP to pyruvate. The active transport performed by the primary and secondary transport systems need energy for the transport (one or two ATP-molecule, or one or more proton(s)), and an additional ATP molecule for the phosphorylation of the imported sugar (77, 148). When, by secondary transport, a sugar molecule is antiported with an end-product instead of a proton, this type of transport is energetically comparable to transport via a PEP:PTS, or it can even cost less energy. PEP:PTSs are often used by (facultative) anaerobic bacteria. Under anaerobic conditions, glycolysis is the only way to produce energy in the form of ATP, since respiration is not possible, and these anaerobic bacteria (even more than aerobic bacteria) have to use this energy efficiently (75, 148).

Carbohydrate transport systems and further carbohydrate metabolism in L. lactis

Sugar transport has been studied in *L. lactis* for more than 30 years. *L. lactis* can import various sugars (188), of which the most common ones are depicted in Table 1. More recently, as a consequence of the increase in genome information, the genes of several transporters have been annotated, occurring either on the chromosome or on plasmids and some of the encoded proteins have been studied in more detail (15, 122). *L. lactis* is a facultatively anaerobic bacterium: it tolerates low levels of oxygen. It can use different carbohydrates as carbon source (70), all of which are degraded to pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 2). The pyruvate produced has several alternative fates depending on the environmental and intracellular conditions.

TABLE 1: Carbohydrate transport systems of *L. lactis*

Transport system	Gene(s) involved	Reference
PST-transporters		
Fructose-PTS	<i>fruA</i>	(9)
Sucrose-PTS	<i>sacB</i> previously <i>scrA</i>	(104, 193, 197)
Cellobiose/Glucose-PTS	<i>ptcBAC</i>	(141)
Mannose/Glucose-PTS	<i>ptnABCD</i>	(195)
Trehalose-PTS	gene unknown	(4)
Lactose-PTS	<i>lacFE</i>	(37, 189)
Galactose-PTS	gene unknown	(190)
Non-PTS transporters		
Maltose permease	<i>malk</i>	(5, 90)
Galactose permease	<i>galP</i>	(54, 190)
Glucose permease	gene unknown	(141)
Trehalose	gene unknown	(4)

The first steps in the metabolism of glucose, galactose and lactose all lead to the production of glucose-6-phosphate (G6P), after which the same route through glycolysis follows (Fig. 2). G6P is converted to fructose-1,6-bisphosphate (FBP) by phosphoglucose isomerase and 6-phosphofructo-1-kinase. The hexose FBP is converted to the triose-phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) by the enzyme fructose-bisphosphate aldolase. The triose-phosphates can be interconverted by triosephosphate isomerase. 3-Phosphoglycerate (3-PGA) is formed from GAP by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After that, 3-PGA is converted to PEP and subsequently to pyruvate by an enolase and pyruvate kinase (PK) respectively. At the level of pyruvate, the metabolic pathway branches.

Although *L. lactis* is generally growing homofermentatively, under less favourable growth conditions, it can perform a mixed-acid fermentation in which besides lactate also acetate, ethanol, 2,3-butanediol and formate are produced (30, 67, 112). Anaerobic conditions combined with fast growth lead mainly to the end product lactate formed from pyruvate by lactate dehydrogenase (LDH). Under certain circumstances some side-products may be formed (Fig. 2).

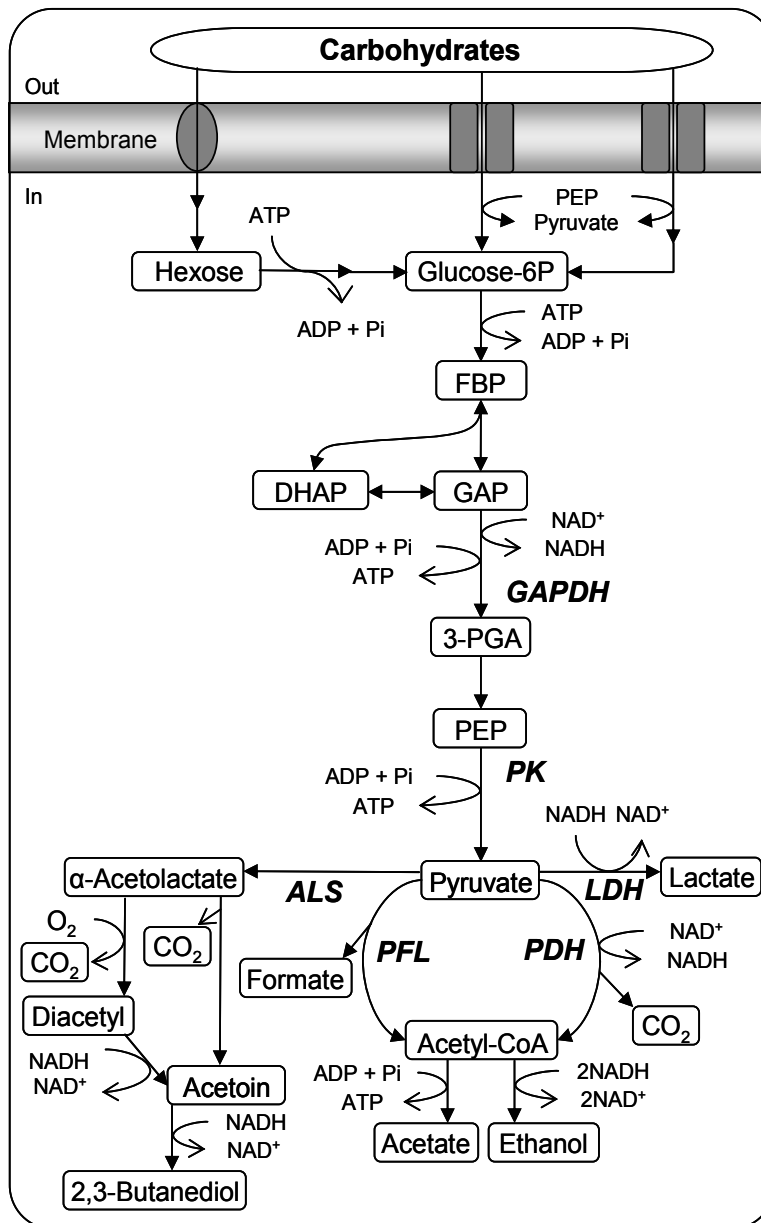


Figure 2:
Schematic
overview of the
fermentation of
carbohydrates
by *L. lactis*.

Carbohydrates can be imported via PTSs or via non-PTS permeases, after which they are converted to glucose-6-phosphate. Glucose-6-phosphate enters the Embden-Meyerhof-Parnas pathway which results in the conversion to, mainly, lactate. The points of formation or expenditure of ATP and NADH are depicted as well as some of the enzymes involved.

Abbreviations: FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 3-

PGA, 3-phosphoglycerate; PEP, phosphoenol-pyruvate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate; NAD⁺, nicotinamide adenine nucleotide; NADH, dihydronicotinamide adenine dinucleotide; CO₂, carbon dioxide; O₂, oxygen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; PDH, pyruvate dehydrogenase; ALS, α-acetolactate synthase.

When the growth conditions of the bacterium are not optimal (e.g. when glucose is limited or when the cells are grown on a less favourable sugar), pyruvate can also be used as substrate for pyruvate formate lyase (PFL), which leads to the production of formate, acetate and ethanol. When *L. lactis* is grown in the presence of (small amounts of) oxygen, pyruvate can be used as substrate for pyruvate dehydrogenase (PDH) and, in that case, carbon dioxide is produced besides acetate and ethanol. When pyruvate accumulates, it can also be converted to 2,3-butanediol or, when small amounts of oxygen are present, to diacetyl, since the affinity of α -acetolactate synthase (ALS, the first enzyme necessary to produce diacetyl or 2,3-butanediol from pyruvate) for pyruvate is very low (176).

The control of the flux through glycolysis in *L. lactis* has been studied extensively over the last decades. Glycolytic intermediates have been determined using different methods and growth conditions, and several glycolytic enzymes and their effectors have been described (122).

Almost all sugar substrates added to *L. lactis* are ultimately recovered as end-products, showing that glycolysis is mainly used to generate energy (75). During fermentation two netto ATP molecules are gained. The ratio of NADH/NAD⁺ is balanced when lactate is formed. Under anaerobic conditions, glycolysis is the only way to produce energy in the form of ATP, since respiration is not possible. Some *L. lactis* strains are able to grow very well aerobically in later states of growth, when haem is provided using a respiratory pathway, but this is mainly beneficial for strains growing on plants or animals (53, 157). Although the glycolytic pathway for the metabolism of sugars in *L. lactis* has been studied widely (75, 122, 152, 192), a detailed understanding of glucose transport and the initial steps in glucose degradation is missing. As shown in this thesis, the first steps in glucose metabolism of *L. lactis* are crucial for the characteristics of fermentation.

The first steps in glucose metabolism

Glucose is taken up and phosphorylated by *L. lactis* via a PEP:PTS, or it is imported via a non-PTS permease, and phosphorylated by glucokinase (Fig. 3). The PTS is considered to be the main route for glucose metabolism (36, 148, 191). Recently, we have shown that *L. lactis* can import glucose via two different PTSs, the mannose/glucose-PTS (EII^{man/glc}), encoded by *ptnABCD*, and the cellobiose/glucose-PTS (EII^{cel/glc}), encoded by *ptcBAC* (this Thesis, Chapter 2, (141)). EII^{man/glc} consists

of a cytosolic EIIAB protein and an integral membrane EIICD protein, while $EII^{cel/glc}$ consists of a cytosolic EIIAB protein and the membrane protein EIIC.

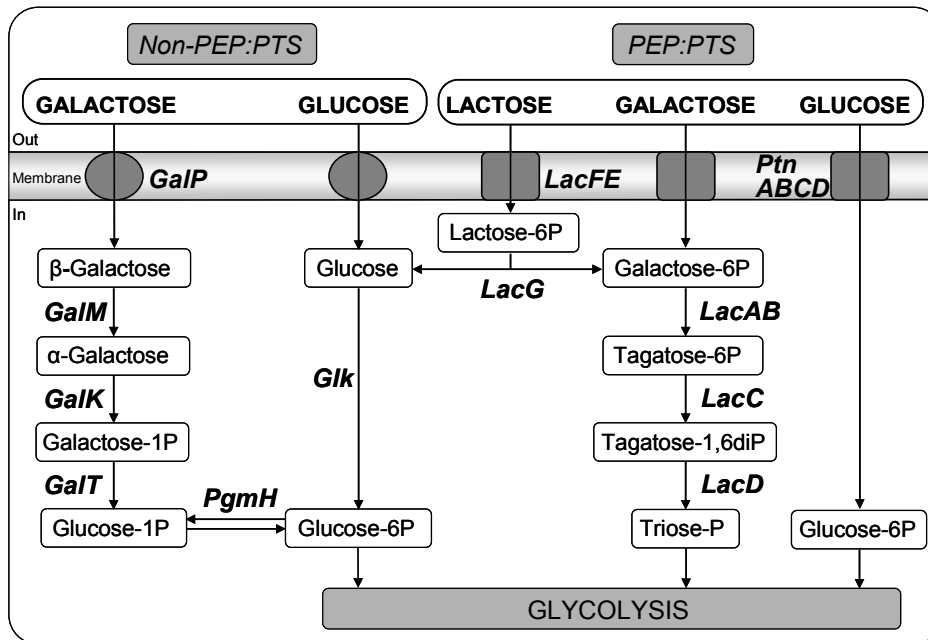


Figure 3: The first steps in glucose, galactose and lactose metabolism in *L. lactis*.

On the left, the non-PEP:PTS transporters for galactose and glucose (transporter unknown) are shown. GalPMKT belong to the Leloir pathway. The PEP:PTS transporters are depicted on the right. LacABCD belong to the tagatose-6P-pathway. The capital P in some of the metabolites signifies phosphate. Abbreviations: GalP, Galactose permease; GalM, galactomutarotase; GalK, galactokinase; GalT, galactose-1-phosphate uridylyltransferase; PgmH, α -phosphoglucomutase; Glk, glucokinase; LacFE, EII^{lac} ; LacG, phospho- β -galactosidase; LacAB, galactose-6-phosphate isomerase; LacC, tagatose-6-phosphate kinase; LacD, tagatose-1,6-diphosphate aldolase; PtnABCD, $EII^{glc/man}$.

When glucose is imported via a non-PTS permease (of which the encoding gene(s) is/are not known yet), it is phosphorylated to G6P by glucokinase (encoded by *glk*). The glucokinase of *L. lactis* is an ATP-dependent protein of 33.8 kD (based on the nucleotide sequence of *glk*), which is able to phosphorylate intracellular glucose (195). The protein has three domains: an ATP-binding site at the N-terminus of the protein, a NagC-domain (regulation of the use of N-acetylglucosamine) spanning almost the complete protein and a ROK-motif (repressor, QRF, kinase) in the middle

of the protein. Proteins belonging to the ROK-family known so far are bacterial sugar kinases, transcriptional repressors, or have as yet uncharacterized functions (196). Proteins having a NagC-domain usually also have a ROK-domain and most of these are also sugar kinases and transcriptional regulators.

Glucokinases from the Gram-positive bacteria *Streptomyces coelicolor* (86), *Staphylococcus xylosus* (214), *Bacillus megaterium* (177) and *Corynebacterium glutamicum* (134), which also contain a ROK-motif, contribute to carbon catabolite repression (CCR), although the precise role in CCR remains unknown (CCR will be discussed later in this Chapter). *E. coli* NagC and Mlc, which are sugar-specific regulatory proteins (140, 166), belong to the ROK-family of proteins and both have an additional almost identical DNA-binding motif. Nevertheless, NagC coordinates the metabolism of aminosugars while Mlc regulates genes involved in sugar uptake together with the cAMP/CAP complex (139). Glk proteins without a ROK-motif have so far not been shown to fulfil regulatory functions. The main role of glucokinase in *L. lactis* is thought to be phosphorylation of glucose, derived from glucose uptake or from the intracellular hydrolysis of disaccharides, such as lactose (195).

The first steps in galactose metabolism

Galactose is imported in *L. lactis* by galactose permease (GalP), a secondary transport system (symport) that couples galactose uptake to sodium translocation (54, 142). The imported galactose is converted to glucose-1-phosphate (G1P) via the Leloir pathway (Fig. 3), comprising steps catalyzed by galactose mutarotase (GalM), galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT) and UDP-galactose-4-epimerase (GalE) (54). The next step is a reversible reaction converting G1P to G6P, which is catalyzed by α -phosphoglucomutase (PgmH). G6P then enters glycolysis (Fig. 2). The *L. lactis* MG1363 *gal* genes are clustered in an operon (*galPMKTE*) (54). The gene encoding α -phosphoglucomutase in *L. lactis* MG1363 has recently been identified (this Thesis, Chapter 5, (121)).

Besides through the Leloir pathway, *L. lactis* can possibly metabolize galactose via the tagatose-6-phosphate pathway. In this case, galactose uptake is most likely mediated by a PEP:PTS (187). The genes for this putative system have not been identified yet but they might be *lacFE*, the genes that specify the lactose-PTS (see below). Whether the Leloir pathway or the tagatose-6-phosphate pathway is primarily used, is strain-dependent (187). *L. lactis* ssp. *cremoris* MG1363, the laboratory strain used in this study, mainly uses the Leloir pathway for galactose fermentation (54).

The first steps in lactose metabolism

L. lactis is able to ferment the milk sugar lactose, which consists of a galactose and a glucose moiety. The disaccharide lactose is imported and phosphorylated by a lactose-PTS encoded by *lacFE*, and is hydrolyzed to glucose and galactose-6-phosphate (Gal6P) by phospho- β -galactosidase (LacG) (Fig. 3). Glucose, the preferred sugar for *L. lactis*, is subsequently phosphorylated to G6P by glucokinase and further metabolized, while Gal6P is further degraded to the triose-phosphates GAP and DHAP by the tagatose-6-phosphate pathway enzymes galactose-6-phosphate isomerase (LacAB), tagatose-6-phosphate kinase (LacC) and tagatose-1,6-diphosphate aldolase (LacD). The lactose metabolic genes are present in an operon (*lacABCDFEG*, (210)) on, among others, lactococcal plasmid pMG820 (106), a derivative of the lactose/proteinase plasmid pLP712 of *L. lactis* NCDO712 (the parental strain of *L. lactis* MG1363).

Besides being metabolized via the tagatose-6-phosphate pathway, some of the Gal6P is probably dephosphorylated and expelled into the medium as a consequence of CCR, a fact that will be discussed later in this chapter, and in Chapter 2 of this Thesis. When the glucose in the cell is depleted, catabolite repression is relieved and galactose can be used. The external galactose is then probably imported by a galactose permease and further metabolized through the Leloir pathway mentioned before.

Regulation by HPr

Bacteria will choose a sugar substrate from the medium in a hierarchical manner. They use the substrate that yields maximum benefit of growth first (21, 56). Metabolic pathways for less favourable sugars are downregulated via so-called Carbon Catabolite Repression, CCR, which combines global transcriptional control with inducer exclusion (202). The phosphorylation state of HPr plays a crucial role in CCR in low-GC Gram-positive bacteria like *L. lactis*, whereas EIIA^{glc} fulfills a similar role in enteric Gram-negative bacteria (202). Here, CCR in Gram-positive bacteria will be further described. HPr can be phosphorylated either at histidine 15 by EI, resulting in HPr-His-P, or at serine 46 by a unique HPr kinase/phosphatase (HPrK/P) (155), resulting in HPr-Ser-P (Fig. 4). HPr-His-P is involved in the phosphate transfer cascade of the PEP:PTS, required for uptake and phosphorylation of sugars, while HPr-Ser-P is involved in the regulation of sugar uptake. HPr-Ser-P is able to exclude uptake of less favourable sugars by inhibition of specific (PTS and non-PTS) sugar transporters while it also activates the global

transcriptional regulator CcpA (carbon catabolite protein A) by binding to it, all inducing CCR (43, 161, 182, 221). HPr-Ser-P is also able to stimulate inducer expulsion (discussed later). The ratio of HPr-His-P and HPr-Ser-P, which is mainly controlled by HPrK/P, depends on the available nutrients and determines which sugar will be used first. When a favourable sugar is used, sugar metabolism in the cell is fast, and high levels of FBP and ATP accumulate, which stimulates the kinase activity of HPrK/P and leads to phosphorylation of HPr to HPr-Ser-P. When a less favourable sugar is used or when glycolysis is progressing, FBP- and ATP-levels go down and inorganic phosphate (Pi) is formed, which inhibits the kinase activity of HPrK/P, resulting in a higher availability of HPr for the PEP:PTS (42, 81).

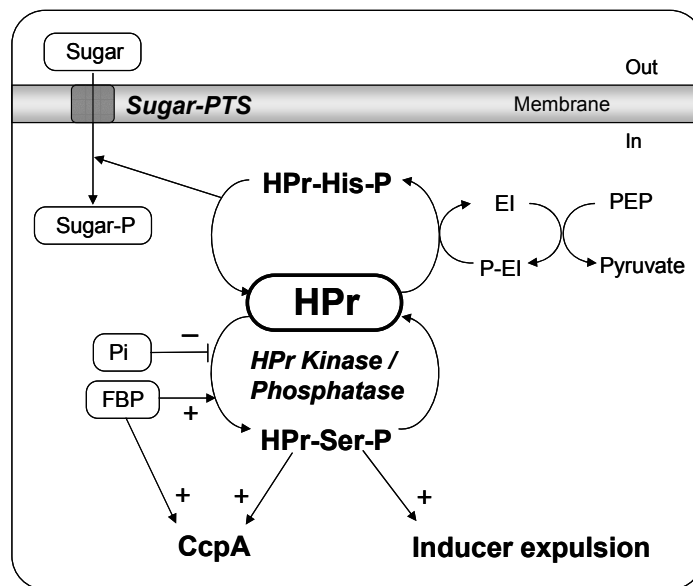


Figure 4: Schematic overview of the role of the histidine protein (HPr) in Gram-positive bacteria. HPr can be phosphorylated at histidine 15 (HPr-His-P) or at serine 46 (HPr-Ser-P). Abbreviations: EI, Enzyme I; PEP, phosphoenolpyruvate; FBP, fructose-1,6-bisphosphate; Pi, inorganic phosphate; CcpA, carbon catabolite protein A; +, activation; -, inhibition.

Inducer expulsion and inducer exclusion

In some cases uptake and hydrolysis of a disaccharide (e.g. lactose) can result in a favoured hexose and a less favoured hexose-phosphate. A mechanism to verify that only the favoured sugar is metabolized is inducer expulsion, which reduces the amount of intracellular sugar phosphates (10, 141, 198). In *L. lactis*, a constitutively

expressed broad-range hexose-6-phosphate hydrolase has been characterized that is held responsible for the dephosphorylation of sugars (194). Furthermore, an inducible hexose-6-phosphate hydrolase has been discovered that can be activated by HPr-Ser-P (220). The genes encoding both enzyme activities have not been identified and, also, the mechanism by which the unphosphorylated sugars are expelled into the medium is as yet unknown.

Another regulatory mechanism which ensures that the most favoured sugar is used first is called inducer exclusion (160). HPr-Ser-P is involved in inducer exclusion in LAB. In *L. lactis*, for example, the uptake of ribose and maltose is strongly inhibited by HPr-Ser-P when glucose is present (116). When *ptsH* is mutated at serine 46, so that HPr-Ser-P can not be formed, glucose inhibition is relieved, suggesting a role for HPr-Ser-P in the inducer exclusion of ribose and maltose (116). In Gram-negative bacteria, EIIA^{glc} directly binds and inhibits the transporter of the less favoured sugar, e.g. it inhibits the maltose transport system of *E. coli* by binding to MalK (39). It is assumed that in low-GC Gram-positive bacteria like *L. lactis* this exclusion mechanism functions in a homologous way to that in Gram-negative bacteria, with HPr-Ser-P being used instead of EIIA^{glc} (42).

Regulation by CcpA

The carbon catabolite protein CcpA, first discovered in *Bacillus subtilis* (59, 63, 114), is also present in *L. lactis* (105). CcpA is a transcriptional regulator protein that binds to a specific DNA-sequence called a *cre* box (catabolite responsive element). A *cre* box can be present within the promoter region or in the coding region of a target gene. The consensus sequence for the *cre* box in *B. subtilis* is WWTGNAARCGNWWCAWW (N, W and R represent any base, A or T, and G or A, respectively) (114). Recently, the functional *cre* sites in *L. lactis* were analyzed, resulting in a *cre* consensus of WWGWAARCGYTWWM (Y and M stand for bases C or T, and A or C, respectively) for *L. lactis* (225). This sequence is, thus, very similar to the consensus *cre* sequence of *B. subtilis*. In *B. subtilis*, the binding of CcpA to its DNA-targets depends on the presence of activated cofactors, like HPr-Ser-P and FBP. Glucose triggers phosphorylation of HPr at serine 46 and binding of HPr-Ser-P to CcpA stimulates DNA-binding of CcpA (103). High concentrations of FBP, as a result of high glycolytic flux, also activate CcpA. CcpA is a pleiotropic regulator protein: it can function as a repressor (e.g. of the *gal*-operon) as well as an activator (as shown for the *las*-operon) in *L. lactis* (105). The genes controlled by

CcpA have been identified in *B. subtilis*. Among the repressed genes were those of the TCA-cycle, while the glycolytic genes were induced (119, 203, 223). The genes involved in the synthesis of glutamate were also upregulated by CcpA, showing that a link exists between carbon and nitrogen metabolism (47). CcpA is also involved in global regulation of sugar metabolism in *L. lactis* (105). *L. lactis* CcpA was recently found to intertwine the regulation of carbon and nitrogen metabolism by regulation of *pepQ*, a gene located in a tail-to-tail arrangement to *ccpA* (55, 225), as was suggested for the CcpA-like protein PepR1 of *Lactobacillus delbrueckii* (165).

Regulation of glycolysis by metabolites

Besides regulation by HPr-Ser-P and CcpA, control of the glycolytic pathway in *L. lactis* also takes place at several points by glycolytic metabolites (Fig. 5).

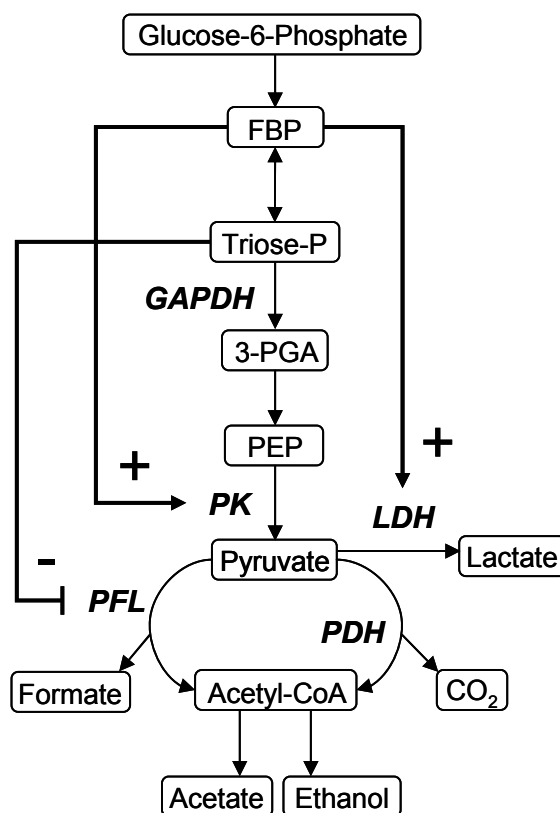


Figure 5: Schematic overview of the regulation of the glycolytic flux by metabolites in *L. lactis*.

Abbreviations: FBP, fructose-1,6-bisphosphate;
Triose-P, triose-phosphates;
3-PGA, 3-phosphoglycerate;
PEP, phosphoenolpyruvate;
CO₂, carbon dioxide;
GAPDH, glyceraldehyde-3-phosphate dehydrogenase;
PK, pyruvate kinase;
LDH, lactate dehydrogenase;
PFL, pyruvate formate lyase;
PDH, pyruvate dehydrogenase;
+ , activation;
- , inhibition.

FBP is one of those regulating metabolites. A high level of FBP is known to activate LDH and PK, while a high level of inorganic phosphate together with a low FBP-concentration (e.g. when the cells are starved) inhibit PK (109, 153, 186, 191, 199). Inhibition of PK leads to accumulation of PEP and 3PGA (152). The metabolism of pyruvate is also influenced by concentrations of the glycolytic intermediates FBP, GAP, and DHAP. Under anaerobic conditions, LDH and PFL compete for pyruvate. During fast growth, high levels of FBP are present that activate LDH, while the high levels of DHAP and GAP under these conditions inhibit the activity of PFL. As a consequence mainly lactate is formed under these conditions. When the bacterium uses a less favourable sugar, or growth rates are lowered by any other cause, less FBP, DHAP, and GAP accumulate, releasing PFL inactivation and allowing the enzyme to metabolize some of the pyruvate to formate, acetate and ethanol. Regulation of glycolysis by redox- and energy-state has also been described. It has been suggested that the intracellular NADH/NAD⁺-ratio controls the flux through glycolysis mainly via the activity of GAPDH producing NADH (50), while other studies measuring metabolites *in vivo* suggested a more important role for the ATP/ADP/Pi pool of the cells (127, 132). Furthermore, which type of regulation plays the major role may be strain-dependent, as was shown for the regulation of LDH enzymes of different strains of *L. lactis* (209).

In vivo NMR spectroscopy

A large part of the results described in this thesis was obtained by *in vivo* Nuclear Magnetic Resonance (NMR). NMR spectroscopy is a powerful analytical technique that makes use of the intrinsic magnetism of some atomic nuclei. When placed in a stationary magnetic field those nuclei (non-zero spin) become aligned with, or against, the field in two energy states. A magnetic resonance (transitions of the nuclei between the states) is induced when the sample is excited by a pulse of radio-frequency radiation. The transition to the lower energy state results in a resonance that can be detected by spectroscopy at a specific frequency. The frequency at which the resonance is detected depends on the specific microenvironment of the nucleus. The electrons surrounding the nucleus of the atom under study and the electrons of nearby atoms are also influenced by the magnetic field, which in turn affects the magnetic field experienced by the nucleus. This means that the same atom might resonate at a different frequency during NMR spectroscopy depending on its surrounding environment. In this way, different molecules can be identified. The separation of resonance frequencies is termed the

“chemical shift” and is expressed in parts per million (ppm). Thus, an NMR spectrum usually consists of several such signals, positioned according to their chemical shifts. As NMR is a quantitative technique, the area of a particular resonance (calculated by integration) arising from a molecule X is proportional to the concentration of X in the sample. Biologically relevant atoms of which the nuclei have a net spin and, therefore, can be detected by NMR are for example ^1H , ^{13}C , ^{15}N and ^{31}P . Not all of these atoms are highly abundant in nature; the natural abundance of ^{13}C for example is only 1.1%. Compounds of interest can be isotopically enriched for ^{13}C at one specific carbon atom. With this technique it is possible to follow the fate of this individual carbon atom through different metabolic pathways in a non-invasive, non-destructive way.

NMR is mainly used as an analytical technique by (bio)-chemists to elucidate the structure of molecules. Using NMR in living microorganisms, for example to follow changes in the concentrations of metabolites, became appealing with the development of more powerful magnets. Nevertheless, already in 1972, when the techniques for ^{13}C -enrichment had improved, NMR was used to trace ^{13}C -enriched metabolites *in vivo* (44). All NMR experiments with living cells described in this Thesis were performed with Ana Rute Neves in the group of Helena Santos (ITQB, Oeiras, Portugal). This team optimized the *in vivo* NMR technique to study sugar metabolism in *L. lactis* over the last decade (122, 124, 152). Great focus was given to the regulation of glycolysis and the application of NMR to identify metabolic bottlenecks and direct metabolic engineering strategies. A main drawback of NMR is its low sensitivity, which limits *in vivo* observations to metabolites present at relatively high concentrations (mM range). Therefore, the majority of NMR experiments are conducted with thick suspensions of non-growing cells. To maintain the cells under specific conditions during the NMR experiment we use a circulating system for on-line NMR. A concentrated cell suspension of non-growing cells in the mid-exponential phase of growth is used in a 50 ml mini-fermenter (Fig. 6).

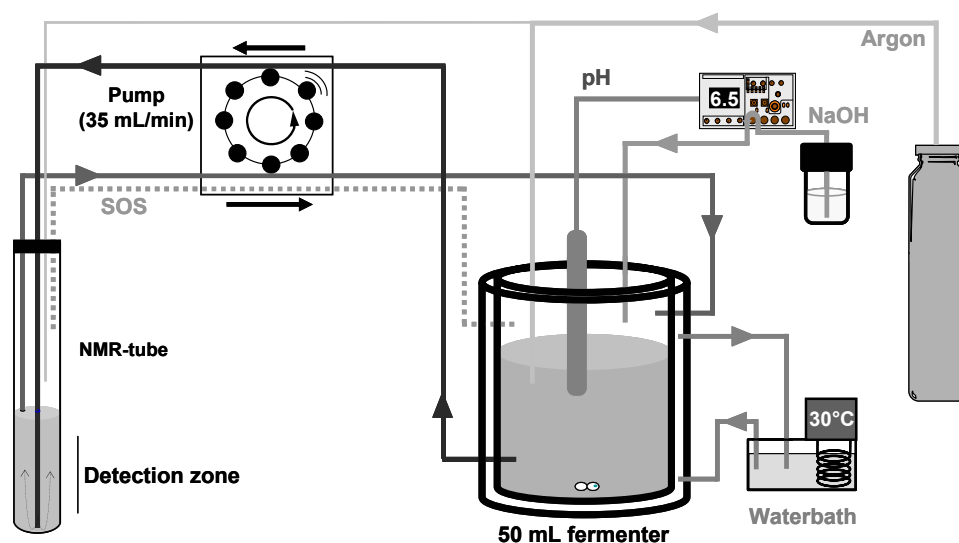


Figure 6: Schematic overview of an *in vivo* NMR experimental setup.

The cells circulate through the system (between the fermenter and the NMR tube) at a speed of 35 ml/min. An "SOS tube" is present to avoid overflow. The temperature, pH and anaerobicity of the cell suspension are controlled. This figure was adjusted from (120).

The cells are circulated between the mini-fermenter and the NMR tube at a speed of 35 ml/min. The cells are constantly stirred in the fermenter and are kept under anaerobic conditions, by flushing with argon gas. The pH of the culture is automatically controlled between 6.45 and 6.55, by titration with a concentrated solution of NaOH. The cells are kept at a constant temperature of 30°C. Circulation was started, an initial spectrum (30 s) was acquired, and ^{13}C -labeled sugar was supplied at a time designated zero (final concentration 20 mM or 40 mM). The time-course for substrate consumption, product formation, and build-up of the pools of intracellular metabolites was monitored online with a time resolution of 30 s. The fermentation of the substrate is followed *in vivo* until no further changes in end-products and intracellular metabolites are detected, after which the spectra are analyzed in detail (124).

DNA-microarray analysis

The expanding miniaturization in the laboratory, the use of pipetting-robots and the growing computer power has resulted in an exponential growth of the number of sequenced genomes. The DNA-microarray technique has become a very important tool for whole genome transcription analysis. With DNA-microarrays the transcriptional profile of at least two different strains, or cells under different conditions, or at different points in time can be compared. Amplicons of all genes of *L. lactis* IL1403 were spotted *in duplo* on a glass slide, on which labelled cDNA can hybridize. In the studies presented in this Thesis two different *L. lactis* strains were compared and differentially expressed genes were made visible. Total RNA was isolated from cells growing in mid-exponential phase, after which cDNA was produced from the RNA. The cDNA was labelled with a Cy3 or Cy5 label, and hybridized to the slides. The whole experiment was performed *in triplo* and the labelled cDNA was hybridized to a total of six slides, using Cy3 and Cy5 dye-swaps (Fig. 7) (83).

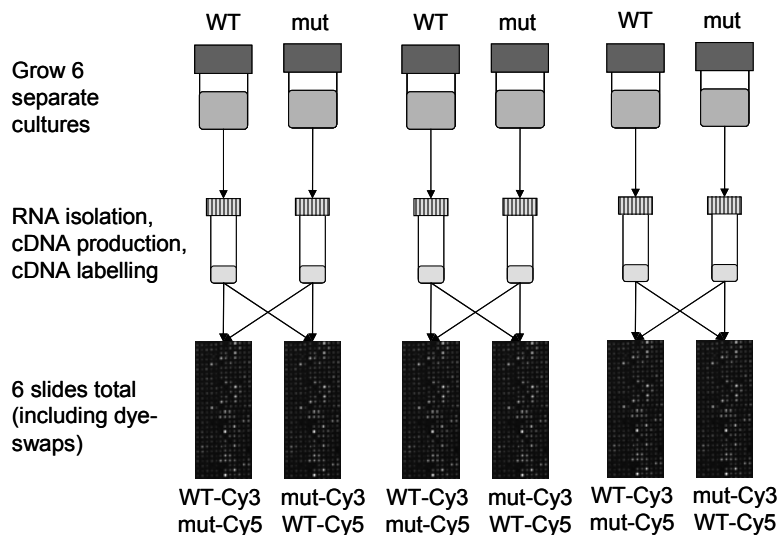


Figure 7: Experimental setup of a DNA-microarray experiment.

Two situations (e.g. wildtype strain (WT) versus mutant (mut)) are compared. The cells are grown in triplo. After RNA isolation, cDNA production and cDNA labelling, the labelled cDNA is hybridized to the DNA-microarray using Cy3 and Cy5 dye-swaps. This methodology results in 6 slides, each containing duplicate spots of each gene.

Since all genes are present *in duplo* on each slide, for each gene a total of twelve spots can be analyzed. The spots on the slides were processed and normalized using software developed by our laboratory (208). Differentially expressed genes were selected using a variant of the paired t-test on normalized ratio data (101).

Nutra Cells

The work described in this thesis was part of an RTD-project sponsored by the European Commission through contract QLK1-CT-2000-01376 (acronym: Nutra Cells). This project was concerned with the development of health-promoting components in food as a result of bacterial activity. The title "Nutra Cells" is based on the term "Nutraceuticals", which was put together from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice (20). Nutraceuticals can be defined as a wide range of foods and food components with a claimed medical or health benefit. In the "Nutra Cells" project, ten academic and industrial partners aimed to improve the nutritional value of foods by engineering micro-organisms for nutraceutical production during food fermentation processes or by addition of these nutraceuticals as ingredients (65).

In this EU-project the trehalose producing *Propionibacterium freudenreichii* ssp. *shermanii* NIZO B365 was shown to produce high levels of trehalose, even when grown in skim milk (27). Trehalose is a low-calorie sugar for humans and could therefore replace high-calorie sugars like sucrose. Another topic of interest was the removal of raffinose and other α -galacto-oligosaccharide sugars from soy-products, since these sugars cannot be metabolized by humans and lead to digestive inconveniences. *Lactobacillus fermentum* CRL722 was found to produce a thermostable α -galactosidase that is able to remove these raffinose-type sugars when added to the process as a purified protein or when expressed *in situ* in an *L. lactis* host (28, 93, 94). Also the α -galactosidase in *Lactobacillus plantarum* ATCC 8014 was characterized (174). Besides sugar engineering work, several groups have obtained promising results in the production of vitamins by microorganisms. In *L. lactis*, the pathways for the production of folate (vitamin B11) and riboflavin (vitamin B2) were studied (23-25, 184, 185). Further engineering resulted in a multivitamin-producing *L. lactis* strain, synthesizing both folate and riboflavin (183). The work described in this Thesis aimed at removing galactose and lactose from dairy fermentation products by engineering of *L. lactis* in an effort to help individuals suffering from lactose intolerance or galactosemia (see below). Furthermore, an effort was undertaken to make *L. lactis* produce glucose from lactose, to reduce the

residual lactose in the end-product and to use the glucose produced as a natural sweetener. Fundamentally, we were interested in the first steps of glucose, lactose and galactose metabolism in *L. lactis* and in the regulatory effect of the use of the different transporters on the rest of sugar metabolism.

Galactosemia

In healthy people, galactose is metabolized in a way comparable to that in bacteria like *L. lactis* and also the names of the human enzymes correspond to their bacterial counterparts (17). Galactose is first imported in human liver cells by a permease, or galactose is formed inside the cell from lactose hydrolysis. The next step is to phosphorylate galactose to Gal1P via galactokinase (GalK). Gal1P is further metabolized to UDP-galactose by galactose-1-phosphate uridylyltransferase (GalT). The interconversion of UDP-galactose and UDP-glucose is catalyzed by uridyl diphosphogalactose-4-epimerase (GalE), after which UDP-glucose can enter glycolysis. Galactosemia is an autosomal recessive inherited enzyme deficiency, with an incidence of about 1 in 35,000 humans in Europe (18, 62, 170). The most common form is called classical galactosemia, which is caused by a deficiency in GalT (17, 98). Other types of galactosemia are rarer and are a result of a deficiency in GalK (113, 133, 164) or GalE (201). Individuals suffering from classical galactosemia are not able to further metabolize Gal1P. Product-inhibition of the kinase results in accumulation of free galactose, which can be converted to either galactitol (206) or galactonate (33), which together with Gal1P can negatively affect different organs. Patients with galactosemia can suffer from cataracts in the eyes, ovarian failure and neurological problems such as cerebral edema, progressive decline in IQ and speech difficulties (158). The severity of the disease can be influenced by the genotype, the height of galactose intake, and the amount of galactose produced endogeneously. Galactosemia patients have a life-long dietary restriction of dairy products and have to exercise care in consuming fruits and vegetables. If LAB used in dairy industry could be engineered in such a way that the residual galactose and lactose in the end-products could be minimized to close to zero, people suffering from galactosemia could use these dairy products in their diet.

Lactose intolerance

About four billion people are unable to digest the milk-sugar lactose properly (26). These lactose intolerant individuals miss the enzyme lactase, which should be

produced in the small intestine. Lactase deficiency leads to discomfort when milk products are digested. The presence of lactose in the small intestine causes water resorption, leading to nausea, cramps and diarrhoea. The anaerobic bacteria present in the large intestine are able to digest the left-over lactose, which leads to toxin formation and gas production (26). The severity of the symptoms depends on the amount of lactose an individual can tolerate and on the person's age and ethnicity. After reaching the age of two, humans start to produce less lactase. Lactose intolerance is very common in the Asian and African population, while the condition is least common among the northern European population. Exclusion of milk and dairy products from the diet is a suitable way to overcome the symptoms of lactose intolerance, but this may lead to nutritional problems. Alternatives like using only fully fermented dairy products in the diet, probiotics with bacterial lactase activity (117), or fermented dairy products with low to no residual lactose (this thesis) are currently under investigation.

Outline of the Thesis

In this thesis the first steps in the metabolism of the sugars lactose, glucose and galactose in *L. lactis* MG1363 are investigated. Using various metabolic engineering strategies combined with state-of-the-art *in vivo* NMR technology, the in-depth analysis of the metabolism of the sugars in the engineered strains underpins the importance of the first steps in metabolism with respect to the regulation of the glycolytic flux and the formation of end products.

In **Chapter 2**, the engineering of *L. lactis* for a more efficient use of lactose as a substrate is described. To achieve this, the metabolism of the favoured sugar glucose had to be blocked. This work led to the identification of a glucose-transporting PTS. This system, encoded by *ptcBAC* was not known to transport glucose. The final strain even produced glucose ("natural sweetening strain") as an end-product, since it could not metabolize the glucose moiety of lactose.

Chapter 3 describes the characterization of the different glucose transport systems in *L. lactis* MG1363 by making targeted single and multiple deletion strains. Transport activities as well as enzymatic activities of key metabolic enzymes were measured and end products of fermentation were determined. Thus, the new glucose-PTS discovered in Chapter 2 is compared to the glucose-PTS already known ($EII^{\text{man/glc}}$) and to non-PTS glucose transport. How glucose is metabolized by *L. lactis* depends on the transport system being used.

Besides glucose and lactose metabolism also galactose metabolism has been studied. Galactose consumption by *L. lactis* MG1363 can be improved, as is shown in **Chapter 4**. In this Chapter the bottleneck in the galactose metabolic pathway was shown to be at the level of α -phosphoglucomutase activity. As the lactococcal gene for α -phosphoglucomutase was at the time not known, the gene encoding α -phosphoglucomutase from *S. thermophilus* was overexpressed in *L. lactis*. This improved the metabolism of galactose.

Chapter 5 describes the isolation of α -phosphoglucomutase activity from *L. lactis* MG1363. The α -phosphoglucomutase activity of *L. lactis* is encoded by *yfgH* (hereafter named *pgmH*), which is an essential gene for growth, as shown by a conditional knock-out strain. Interestingly, sequence and biochemical analyses showed that PgmH of *L. lactis* is related to the eukaryotic phosphomannomutases. Finally, **Chapter 6** summarizes the most important results of the work described in this Thesis and puts them in perspective. Directions for future experiments are given in order to further improve the knowledge on sugar metabolism and its regulation in *L. lactis*.

